

FDA Contract 71-331

using the chick embryo as the test system

No Date

Evaluation of chemicals for Toxic & Teratogenic effects

**Ammoniated Glycyrrhizin** FDA 71-1

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WARF INSTITUTE, INC.

MADISON, WISCONSIN

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EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS  
USING THE CHICK EMBRYO AS THE TEST SYSTEM

AMMONIATED GLYCYRRHIZIN: FDA 71-1

WARF INSTITUTE, INC.  
Madison, Wisconsin



FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS  
USING THE CHICK EMBRYO AS THE TEST SYSTEM

Objective: To determine the toxic and teratogenic effects of GRAS List compounds when injected into the air cell and yolk of fertile chicken eggs.

Procedure:

A. Test System and Incubation Procedures:

Fertile hatching eggs were chosen from a single comb white leghorn breeder flock. The eggs were candled and graded to eliminate internal and external defects; blood and meat spots, tremulous air cells, rough or cracked shells. The eggs chosen for injection weighed from 23 - 26 ounces/dozen and were not washed or dipped. The eggs were gathered within 48 hours of the injection or incubation and were held at 50 - 60°F. and 60 - 80% relative humidity. The breeder ration fed the flock was formulated by the breeder to meet or exceed the recommendations of the Nutrient Requirements of Poultry, Number 1 - 1971, National Academy of Sciences, and contained no additions of antibiotics, arsenicals, nitrofurazones or similar chemical additives. The breeder flock was blood tested and negative for pullorum-typhoid and mycoplasma gallisepticum.

Eggs were incubated in Jamesway 1080 forced air incubators equipped with automatic controls to regulate temperature, humidity and egg turning. Temperature and relative humidity were maintained at 99.5°F. and 86°F.\* wet bulb respectively for the first 18 days of incubation and eggs were turned each two hours. The eggs were then transferred to the hatcher in 3½" x 5" x 25" covered hardware cloth hatching baskets for the hatching period. Temperature in

\* 86°F. wet bulb refers to temperature of wet bulb apparatus in standard incubator hatching equipment and is equivalent to approximately 56% relative humidity.



the hatcher was maintained at 98.5oF. and relative humidity at 86oF. wet bulb. When the humidity had risen to 88 degrees as a result of moisture generated by hatching the hatcher was adjusted to hold 88oF. wet bulb relative humidity until the chicks were removed on the morning of the 23rd day of incubation.

Prior to incubating or hatching each setting of eggs and following each hatching the incubator and its metal parts were thoroughly cleaned by vacuuming and washing with a 200 ppm solution of "ROCCAL" which contains 10% alkyl (C12, C14, C16 and related alkyl groups from C8 to C18) - dimethyl benzyl ammonium chloride. The sanitized surfaces were allowed to completely air dry prior to the introduction of eggs. Following each transfer of eggs to the hatching compartment, and when temperature and humidity had returned to normal levels, the hatcher and the eggs it contained were fumigated by combining 10 grams of potassium permanganate crystals and 20 grams of 37% formaldehyde solution.

#### B. Test Sample Preparation and Administration:

The test sample was taken up in an appropriate solvent to facilitate administration at the levels chosen. Sterile glassware, syringes and needles were employed to prepare and administer the test sample or solvent. Eggs previously selected were candled and the location of the air cell marked with pencil. The eggs were then randomized into the experimental groups. Injection of solvent or test sample dilution was accomplished by placing the material on the air cell membrane or by injection into the yolk sac. These administrations were made at both 0 and 96 hours of incubation. For the 0 hour experiments the eggs were assumed to be fertile; however, in the 96 hour experiments the eggs were candled as previously described and only those eggs with a well developed 96 hour embryo were selected for use.

##### 1. Air Cell Administration:

The eggs were wiped at the injection site with 70% ethanol and allowed to air dry. A hole measuring approximately 6mm was then drilled over the air cell in each egg using a "Dremel Moto-tool", model 270. The cutter employed deflected the shell fragments upwards and outwards. Remaining shell membrane fragments were removed with a small



forceps and the surface of the egg membrane visually examined for damage. The solvent or test sample was then deposited on the egg membrane with a model SB2 Syringe Microburet. Immediately following, the hole was sealed with  $\frac{1}{2}$ " Scotch Brand transparent tape. Two additional groups of eggs were normally included with each air cell experiment; a group which had been drilled, shell membrane fragments removed, and sealed only and a group of control eggs which had received no treatments whatsoever.

## 2. Yolk Administration:

The eggs were placed within a Fisher Scientific "Isolator/Lab" equipped with plastic irises through which the hands and forearms were placed during injection. Prior to injection the eggs and miscellaneous required equipment were submitted to a fumigation of 1.8 grams of potassium permanganate crystals and 3.6 grams of 37% formaldehyde. The eggs were held in this atmosphere for 30 minutes prior to further handling.

Each egg was then wiped at the injection site with 70% ethanol and allowed to air dry. A small hole was engraved directly over the air cell with a Burgess model V-13 Vibro-Graver. Care was taken not to damage the membrane attached to the shell. The surface of the egg at the engraved site was vacuumed to remove the shell particles produced. The egg was then slid onto the needle of the Syringe Microburet with the egg horizontal on its long axis until the top of the egg reached the hub of the 1" - 25 ga. hypodermic needle. Following the injection of the material into the yolk sac, the egg was carefully withdrawn from the needle and the hole sealed with transparent tape. The hypodermic needle was carefully wiped with a sterile gauze pad prior to the next injection. As in the air cell administration, normally two additional groups of eggs were included in each yolk experiment; a group which had been drilled, pierced with the hypodermic needle and sealed only and a group of control eggs which had received no treatment other than fumigation.

Following the air cell and yolk injections the eggs were identified as to experiment and group with a No. 3 lead pencil and were then incubated as described above.

**C. Test Profile:**

The work was divided into one or more Preliminary Range Finding Experiments, two Dose-Response and Teratogenic Experiments, and Ancillary Investigations (Post Hatch Trials).

**1. Preliminary Range Finding Experiments:**

The objective of these trials was to locate the approximate LD-50 of the test sample. This data was used to design the dose levels for the Dose-Response trials. The test sample and solvent were administered by two routes; air cell and yolk, and at 0 and 96 hours of incubation. In general, at each route and time of incubation, 5 volumes of test sample dilution were administered together with 5 levels of solvent at the same volumes. Control eggs were also usually included as described above. Normally 10-20 eggs were used per group in these trials. When necessary these trials were repeated in an effort to locate the approximate LD-50 for the test compound.

Beginning on the 6th day of the incubation, the eggs set in the Preliminary Range-Finding Experiments were candled daily and non-viable embryos removed. These embryos were examined grossly for determination of developmental age and evidence of teratogenic effect, however, mortality was the main parameter in these trials. The remaining eggs were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The resultant chicks and non-viable embryos were examined grossly for teratogenic effects and all pertinent data was recorded. An estimate of the LD-50 for the test compound was then made.

**2. Dose-Response and Teratogenic Experiments:**

Based upon information from the Preliminary Range-Finding Experiments, the Dose-Response Experiment was designed, employing 5 levels of sample dilution expected to produce mortality from the background level up through approximately 90%. Five volume levels of solvent were included as solvent controls at each route and time of administration. Normally 10 eggs/group were used in the solvent series with 50 eggs/group for the test sample dilutions. Twenty eggs/group were normally included for the drilled or pierced and non-treated controls. Two such experiments were conducted for each sample so that ultimately 100 eggs were tested on each test dilution at each route and stage of incubation.

The eggs set in these experiments were candled daily beginning on the 6th day of incubation and the non-viable embryos removed for examination as previously described. Where necessary, embryos were examined with the aid of a dissecting microscope. Remaining embryos were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The apparently normal chicks were then removed from the hatching trays and examined externally for anomalies.

Remaining non-viable embryos and chicks which were alive but unable to hatch were individually examined externally for abnormalities. These non-viable embryos, chicks which were alive but unable to hatch, and a portion of the normal chicks were examined in one aspect by X-ray. The chicks and embryos which had been X-rayed and all remaining normal chicks were then examined internally for possible anomalies of the viscera. All pertinent data were recorded.

### 3. Post Hatch Trials:

Apparently normal chicks were chosen from one 50 egg experiment for this portion of the study.

Generally 20 chicks (straight-run) were wing banded from each level chosen and were placed in Jamesway electrically heated battery brooders. Central Soya Chick Starter was fed as the sole ration to 8 weeks of age and Central Soya Grower from 8 weeks of age to termination. These diets were non-medicated. The chicks chosen were usually from the approximate LD-50 and no-effect levels for the test compound from each route of administration and time of incubation. Negative control, untreated chicks, were also included. In some cases chicks were chosen from groups where a relatively high incidence of anomalies were seen rather than from the LD-50 or no-effect levels specifically. Body weight data were collected weekly through 4 weeks of age and bi-weekly to termination. Average group feed consumption was recorded periodically.

### 4. Histopathology:

A random sampling of birds from selected groups were specified for histologic examination. These chicks comprised 5 males and 5 females from the test groups selected and 5 males and 5 females from a negative control group. Groups to be sampled were selected on the basis of observations of specific effects and a judgment made as to what groups would give the most information from the limited histopathologic examination.



The chicks sacrificed were either day old or varying ages in a Post Hatch Trial. The following tissues were collected, trimmed, dehydrated, embedded in paraffin, sectioned and stained with hematoxilin and eosin:

1. Thyroid
2. Liver
3. Spleen
4. Pancreas
5. Lung
6. Heart
7. Kidney
8. Gonad
9. Bursa

The prepared slides were examined and remarkable alterations noted.

#### Results:

The data developed in the testing of ammoniated glycyrrhizin are presented in the following tables:

- Table 1 - Air Cell at 0 Hours
- Table 2 - Air Cell at 96 Hours
- Table 3 - Yolk at 0 Hours
- Table 4 - Yolk at 96 Hours
- Table 5 - Body Weight Data - Males
- Table 6 - Body Weight Data - Females
- Table 7 - Histopathology - Grow-Out Birds
- Table 8 - Gross Pathology - Grow-Out Birds

In Tables 1 through 4, the following comments apply:

Column 1 gives the dose administered milligrams per kilogram, respectively. (The milligrams per kilogram figure is based on an average egg weight of fifty grams).

Column 2 is the total number of eggs treated.



Column 3 is the percent mortality, i.e., total non-viable divided by total treated eggs.

Column 4 is the total number of abnormal birds expressed as a percentage of the total eggs treated. This includes all abnormalities observed and also toxic response such as edema, hemorrhage, hypopigmentation of the down and other disorders such as feather abnormalities, significant growth retardation, cachexia, ataxia or other nerve disorders.

Column 5 is the total number of birds having a structural abnormality of the head, viscera, limbs, or body skeleton expressed as percentage of the total eggs treated. Toxic response and disorders such as those noted for column 4 are not included.

Column 2 through 5 have been corrected for accidental deaths if any occurred. Included in these columns are comparable data for the solvent-treated eggs and the untreated controls.

The mortality data in column 2 have been examined for a linear relationship between the probit percent mortality versus the logarithm of the dose. The results are indicated at the bottom of each table.

The data of columns 3, 4 and 5 have been analyzed using the Chi Square test for significant differences from the solvent background. Each dose level is compared to the solvent value and levels of percent mortality or percent abnormalities that are significant (probability of being the same is 5% or less) are indicated by an asterisk in the tables. All values so indicated have a higher incidence than the solvent values.

#### Discussion:

The comments and data which follow concern the results when ammoniated glycyrrhizin was employed in the test system.

Significant toxicity (p.05) was observed in the 0 hour air cell treatments at 220.0, 293.3, 366.7, 440.0 and 513.3 mg/kg when compared with the solvent control. The calculated



LD-50 was 337.4 mg/kg. At 96 hours air cell, the mortality response was again significant and dose related at levels of 110.0, 220.0, 366.7 and 440.0 mg/kg. The LD-50 was 121.6 mg/kg.

In 0 hour yolk treatment, the mortality response was significantly elevated at doses of 22.0, 44.0, 110.0, 220.0 and 440.0 mg/kg. The LD-50 was calculated at 171.9 mg/kg. At 96 hour yolk, the response was significant at dose levels of 14.7, 22.0, 36.7, 66.0, 73.3, 132.0, 183.3, 220.0 and 366.7 mg/kg. The calculated LD-50 was 86.5 mg/kg.

Significant instances of terata were seen at all treatment times and routes.

In the 0 hour air cell treatments, the primary terata seen were retarded development or dwarfism. In addition clubbed down, hair-like down, long mandible, short mandible, flexed mandible, crossed beak, maxilla absent, microphthalmia, anophthalmia, ablepharia, eyelid dysplasia, comb absent, exencephaly, celosmia, small liver lobe, perosis, rotated hock, light down, edema, bloody yolk, poorly healed navel, malposition and ascites were observed. The terata observed at 293.3 mg/kg, with the exception of the dwarfism, were mainly those of the head involving the eyes and beak.

In the 96 hour air cell treatment, significant occurrences of terata were seen at dose levels of 22.0, 44.0, 110.0 and 220.0 mg/kg. Retarded development was a major contributor to the total terata. Also included were clubbed and hair-like down, short mandible, long mandible, flexed mandible, parrot and crossed beak, eyelid dysplasia, ablepharia, exencephaly, skull bulla, celosomia, kidney absent, perosis, leg and wing micromelia, oligodactyly, short toes, fused toes, wing phocomelia, leg ectromelia, and curled toes. Absence of one or both kidneys was seen at the 4 dose levels mentioned above, and the level of anomalies of the head, viscera and limbs were unusually high at the 98 egg, 110.0 mg/kg dose.

At 0 hour yolk, the instances of terata were significantly elevated at 22.0, 44.0, 110.0, 220.0 and 440.0 mg/kg. Again, retarded development was a major contributor to the total abnormalities. Likewise, clubbed down and light down were found in rather high numbers across the previously mentioned dosages. The remaining abnormalities were low in incidence at any particular dose level and were similar to those seen at 96 hour air cell. The absence of one or both kidneys was not observed.

At 96 hour yolk, the percent total abnormalities was significantly elevated at 36.7, 73.3, 132.0, 183.3 and 366.7 mg/kg. As at 0 hour yolk, the occurrences of retarded development and clubbed or light down were considerable and contributed heavily to the total abnormalities. Other serious abnormalities en-



countered at greater than background levels were: flexed mandible, short mandible, parrot beak, celosmia and edema.

The normal down color for chicks of this breed is pale yellow. It is therefore of particular interest that from 14 to 100 percent of the chicks which hatched at the 0 hour yolk treatments evidenced hypopigmentation of the down. The percent of hatched chicks involved and the severity of the abnormality was dose related. At 440.0 mg/kg, the down was "chalk white" and 100 percent of the hatched chicks were involved. At 96 hour yolk, the percent involvement ran from 5 to 50 percent in the hatched chicks and the hypopigmentation was less severe than seen at the 0 hour yolk treatment.

Moderate dwarfism was a frequent observation in this experiment. To clarify our designation of dwarfism we will explain our classification procedure. At the first candling, day 5 or 6, we did not classify any embryos as retarded unless they were alive and definitely younger in development than 5 or 6 days. In subsequent candling any dead embryo judged by size to be 3 days behind in development was labeled as slight dwarfism, 4 days behind was labeled moderate dwarfism and 5 days or more behind was labeled severe dwarfism. If embryos were removed alive, 1 day behind was labeled slight, 2 days behind was labeled moderate and 3 days behind was labeled severe dwarfism. At hatch time an 18 day embryo was classified slightly dwarfed, a 17 day embryo was classified as moderate dwarfism and a 16 day embryo was classified as severe dwarfism. One might suspect that at the toxic levels administered, embryo development could be delayed due to metabolic or nutritional alterations that produced temporary growth depression which would not result in a permanent growth defect. The retarded development seen was largely moderate in severity and chicks hatched from groups where dwarfism was seen performed normally.

Post-hatch birds were raised to 12 weeks of age and included birds from the untreated control and various treatment groups as indicated in the attached tables. Body weight gains and feed consumption were considered to be within normal limits in all groups. As the chicks with light down became older and their final feathering was established, the feather color was normal.

Histopathological examination was conducted on tissues from birds in the 96 hour air cell - 110.0 mg/kg and 96 hour yolk - 73.3 mg/kg. The tissues were compared with those of



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similar age untreated controls. The tissue alterations were minimal in nature and rather randomly distributed. No kidney alterations were noted.

X-ray examination did not reveal abnormalities not already noted on gross examinations.

## Conclusion:

Under the conditions specified for this trial, ammoniated glycyrrhizin was shown to be particularly toxic at the 96 hour yolk administration and teratogenic in both the air cell and yolk treatments at 0 and 96 hours.

Signed R. M. Bodden

By and For WARF Institute, Inc.

September 16, 1974

Test Sample: Ammoniated Glycyrrhizin

Identification: F.D.A. 71-1

Solvent System: 50% Absolute Ethanol In Sterile Distilled H<sub>2</sub>O

Breeder Flock: N-1

Preliminary Range Finding Experiments

<u>Experiment No.</u>	<u>Initiated</u>
8	12/16/71
21	3/30/72

Dose Response Experiments

<u>Experiment No.</u>	<u>Initiated</u>
27	5/08/72
44	8/28/72



Table 1

Ammoniated Glycyrrhizin  
Air Cell At 0 Hours

Dose Mg/Kg	Number of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
513.3	43	81.39*	65.11*	4.65
440.0	20	60.00*	15.00*	5.00
366.7	110	58.18*	22.72*	5.45
293.3	97	56.70*	26.80*	7.21*
220.0	127	30.70*	17.32*	2.36
146.7	100	14.00	7.00	3.00
110.0	30	20.00	10.00	3.33
73.3	70	12.85	7.14	4.28
36.7	30	16.66	3.33	.00
14.7	10	.00	.00	.00
50% Ethanol	210	12.38	2.38	1.42
Drilled Control	80	17.50	5.00	.00
Control/ Control	260	8.46	4.23	1.15

\*\* LD-50 337.4 mg/kg

\* Significantly different from solvent ( $P \leq 0.05$ )

Table 2  
Ammoniated Glycyrrhizin  
Air Cell At 96 Hours

Dose Mg/Kg	Number of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
440.0	18	100.0*	5.55	.00
366.7	10	100.0*	30.00	10.00
220.0	94	87.23*	41.48*	19.14*
220.0	30	93.33*	23.33*	10.00
110.0	98	58.16*	34.69*	32.65*
110.0	30	30.00	23.33*	3.33
73.3	20	15.00	15.00	.00
44.0	99	16.16	18.18*	13.13*
36.7	30	13.33	3.33	.00
22.0	100	24.00	20.00*	10.00*
14.7	10	10.00	10.00	.00
8.8	100	14.00	3.00	2.00
50% Ethanol	209	14.83	9.09	1.91
Drilled Control	80	5.00	5.00	1.25
Control/ Control	260	8.46	4.23	1.15

\*\*LD-50 121.6 mg/kg

\*Significantly different from solvent ( $P \leq 0.05$ )



Table 3

Ammoniated Glycyrrhizin  
Yolk At 0 Hours

Dose Mg/Kg	Number of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
440.0	50	84.00*	34.00*	2.00
220.0	110	64.54*	31.81*	5.45
132.0	10	40.00	20.00	10.00
110.0	100	46.00*	28.00*	5.00
66.0	10	40.00	20.00	10.00
44.0	99	41.41*	20.20*	3.03
22.0	109	35.77*	24.77*	5.50
8.8	9	22.22	11.11	.00
4.4	50	20.00	4.00	2.00
50% Ethanol	150	20.00	6.66	2.00
Pierced Control	60	20.00	3.33	3.33
Control/ Control	260	8.46	4.23	1.15

\*\*LD-50 171.9 mg/kg

\*Significantly different from solvent ( $P \leq 0.05$ )

Table 4  
Ammoniated Glycyrrhizin  
Yolk At 96 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
366.7	100	60.00*	42.00*	7.00
220.0	10	70.00*	20.00	10.00
183.3	97	70.10*	42.26*	4.12
132.0	10	40.00*	30.00*	.00
73.3	99	47.47*	33.33*	11.11*
66.0	10	10.00*	.00	.00
36.7	100	38.00*	23.00*	4.00
22.0	10	10.00*	.00	.00
14.7	100	33.00*	15.00	4.00
8.8	10	.00*	.00	.00
50% Ethanol	148	8.78	6.75	3.37
Pierced Control	60	16.66	8.33	3.33
Control/ Control	260	8.46	4.23	1.15

\*\*LD-50 86.5 mg/kg

\* Significantly different from solvent ( $P \leq 0.05$ )

Table 5 Body Weight Data - Post Hatch Response  
F.D.A. 71-1: Ammoniated Glycyrrhizin  
(Males)

Test (1) Dose (1)	Time/Route	Average Individual Body Weight - Grams							
		Week 1	2	3	4	6	8	10	12
-	Control/Control	63	126	213	303	537	1002	1145	1438
366.7	0/AC	63	121	198	289	501	894	989	1180
146.7	0/AC	71	127	209	289	477	834	940	1202
220.0	0/Y	67	111	190	267	496	1007	1090	1312
4.4	0/Y	75	144	240	331	558	1049	1160	1460
110.0	96/AC	71	129	210	285	455	919	1035	1228
8.8	96/AC	71	138	225	306	521	1014	1124	1332
73.3	96/Y	72	136	223	304	531	956	1067	1324
14.7	96/Y	68	130	217	312	551	1026	1153	1428

(1) Milligrams/Kilogram of body weight

Table 6 Body Weight Data - Post Hatch Response  
F.D.A. 71-1: Ammoniated Glycyrrhizin  
(Females)

Test (1) Dose (1)	Time/Route	Average Individual Body Weight - Grams							
		Week 1	2	3	4	6	8	10	12
-	Control/Control	66	124	209	298	523	873	986	1213
366.7	0/AC	62	121	199	266	461	850	949	1165
146.7	0/AC	70	127	207	277	440	738	824	1024
220.0	0/Y	64	117	191	259	436	740	819	1248
4.4	0/Y	68	125	205	285	466	781	864	1080
110.0	96/AC	74	130	207	257	444	745	941	1028
8.8	96/AC	66	122	197	267	445	725	803	988
73.3	96/Y	65	123	203	274	467	762	824	1026
14.7	96/Y	65	119	185	254	407	751	849	1032

(1) Milligrams/Kilograms of body weight



Table 7  
Ammoniated Glycyrrhizin  
Histopathology - Grow-Out Birds

<u>Histologic Observations</u>	<u>Negative Control (10)</u>	<u>96/AC 110 MG/KG (9)</u>	<u>96/Y 73 MG/KG (10)</u>
<u>Thyroid</u>			
focal areas of cellular infiltration	1		
area of cellular infiltration under capsule	1		
<u>Lungs</u>			
congested	3		
<u>Heart</u>			
focal areas of cellular infiltration	1		
<u>Spleen</u>			
peripheral pigmentation	8	1	2
diffuse pigmentation (mild)			5
very dense		1	
<u>Liver</u>			
diffuse pigmentation	1	1	1
minimal pigmentation			3
peripheral pigmentation	6	6	2
focal areas of cellular infiltration	9	4	3
degenerate severe diffuse vacuolization		1	2
<u>Proventriculus</u>			
focal areas of cellular infiltration	1		
<u>Pancreas</u>			
degenerate	1		



Table 7 (Continued)  
Ammoniated Glycyrrhizin  
Histopathology - Grow-Out Birds

<u>Histologic Observations</u>	<u>Negative Control (10)</u>	<u>96/AC 110 MG/KG (9)</u>	<u>96/Y 73 MG/KG (10)</u>
<u>Gonads</u>			
testicles immature	1	1	2
testicles degenerate	1	1	3
testicles tubules destroyed			1
testicles numerous irregular tubules			2
<u>Bone Marrow</u>			
numerous fat vacuoles	2		

Table 8  
Ammoniated Glycyrrhizin  
Gross Pathology - Grow-Out Birds

<u>Gross Observations</u>	<u>Negative Control (10)</u>	<u>96/AC 110 MG/KG (9)</u>	<u>96/Y 73 MG/KG (10)</u>
<u>Limb</u> right leg dysarthrosis			1
<u>Kidneys</u> hemorrhage	1		